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A Transcriptional Regulator and ABC Transporters Link Stress Tolerance, (p)ppGpp, and Genetic Competence in *Streptococcus mutans*▽†

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***Streptococcus mutans*, a primary agent of dental caries, has three (p)ppGpp synthases: RelA, which is required for a mupirocin-induced stringent response; RelP, which produces (p)ppGpp during exponential growth and is regulated by the RelRS two-component system; and RelQ. Transcription of *relPRS* and a gene cluster (*SMu0835* to *SMu0837*) located immediately upstream was activated in cells grown with aeration and during a stringent response, respectively. Bioinformatic analysis predicted that *SMu0836* and *SMu0837* encode ABC exporters, which we designated *rcrPQ* (*rel competence-related*) genes, respectively. *SMu0835* (*rcrR*) encodes a MarR family transcriptional regulator. Reverse transcriptase PCR (RT-PCR) and quantitative RT-PCR analysis showed that RcrR functions as an autogenous negative regulator of the expression of the *rcrRPQ* operon. A mutant in which a polar insertion replaced the *SMu836* gene (Δ*836polar*) grew more slowly and had final yields that were lower than those of the wild-type strain. Likewise, the Δ*836polar* strain had an impaired capacity to form biofilms, grew poorly at pH 5.5, and was more sensitive to oxidative stressors. Optimal expression of *rcrPQ* required RelP and vice versa. Replacement of *rcrR* with a nonpolar antibiotic resistance marker (Δ*835np*), which leads to overexpression of *rcrPQ*, yielded a strain that was not transformable with exogenous DNA. Transcriptional analysis revealed that the expression of *comYA* and *comX* was dramatically altered in the Δ*835np* and Δ*836polar* mutants. Collectively, the data support the suggestion that the *rcrRPQ* gene products play a critical role in physiologic homeostasis and stress tolerance by linking (p)ppGpp metabolism, acid and oxidative stress tolerance, and genetic competence.**

Due to the intermittent feeding and diurnal rhythms of the host, the microbial biofilms colonizing the human oral cavity are continually exposed to changes in pH, redox potential, and nutrient source and availability (10). *Streptococcus mutans*, the primary etiological agent of dental caries, gains a competitive advantage over many commensal species because of its ability to thrive under conditions that are favorable for caries development (17, 19). In particular, *S. mutans* is highly effective at producing acids from dietary carbohydrates, which causes a decrease in the pH of oral biofilms and demineralization of the tooth (8, 26). *S. mutans* is also more acid tolerant (aciduric) than many commensals, being able to grow and to carry out glycolysis at pH values that are well below the pH needed to damage tooth mineral. In fact, the property of aciduricity is routinely observed in bacteria that are strongly associated with dental caries (21).

Carlsson first described the lifestyle of oral bacteria as a feast-and-famine existence to illustrate that these organisms are confronted with extended periods during which saliva is the primary nutrient source, interspersed with comparatively short intervals where nutrients from dietary sources are abundant (11). These often dramatic changes in both the type and the availability of nutrients require flexibility in the expression of genes and metabolic potential if oral bacteria are to persist and become numerically significant in dental biofilms. One conserved strategy for bacteria to cope with fluctuations in nutrient availability and other environmental stresses involves the enzymatic production of the nutritional alarmones guanosine 3′-diphosphate, guanosine 5′-triphosphate, and guanosine 3′,5′bispyrophosphate, commonly abbreviated (p)ppGpp (12). An increase in (p)ppGpp pools has global effects on gene expression, causing downregulation of genes for macromolecular biosynthesis and upregulation of genes for protein degradation and amino acid biosynthesis, commonly referred to as the stringent response (42, 46). The stringent response is especially important in bacteria that are exposed to changing environments, as it can help to maintain genomic stability, to avoid an increase in translational errors, and to prevent the depletion of limited energy resources (41, 46).

In many Gram-negative bacteria, there are two enzymes governing (p)ppGpp production: RelA, with strong synthase activity and weak hydrolase activity, and SpoT, with strong hydrolase activity and weak synthase activity (16, 41). Many other bacteria, including most Gram-positive organisms, have a single bifunctional RelA enzyme with both (p)ppGpp synthase and hydrolase activities (33, 43). RelA of *S. mutans* is similar to other Gram-positive RelA/SpoT homologue (RSH) enzymes and appears to be entirely responsible for increased (p)ppGpp production during a mupirocin-induced stringent response (22). However, *S. mutans* has two additional (p)ppGpp synthetases, RelP and RelQ, which can produce (p)ppGpp but which lack the hydrolase domain and the domain for allosteric control of enzymatic activity (22). The discovery of the two additional (p)ppGpp synthetases in *S. mutans* and, subsequently, potential homologues of these proteins in many other species (34) revealed that bacteria have additional

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
UA159	Wild type	
Δ*835np*	Δ*SMu0835*::NPKm^r	This study
Δ*835p*	Δ*SMu0835*::ΩKm^r	This study
Δ*836np*	Δ*SMu0836*::NPKm^r	This study
Δ*836p*	Δ*SMu0836*::ΩKm^r	This study
Δ*837np*	Δ*SMu0837*::NPKm^r	This study
Δ*835-837np*	Δ*SMu0835* Δ*SMu0836* Δ*SMu0837*::NPKm^r	This study
Δ*836-837np*	Δ*SMu0836* Δ*SMu0837*::NPKm^r	This study
835$^+$/Δ*835np*	Δ*SMu0835*::NPKm^r harboring pDL278-*SMu0835*	This study
835$^+$/Δ*835p*	Δ*SMu0835*::ΩKm^r harboring pDL278-*SMu0835*	This study
835$^+$/Δ*835-7np*	Δ*SMu0835-7*::NPKm^r harboring pDL278-*SMu0835*	This study
835$^+$/*wt*	UA159 harboring pDL278-*SMu0835*	This study
Δ*tpx*	Δ*tpx*::NPKm^r	This study
Δ*relA*	Δ*relA*::Erm^r	22
Δ*relP*	Δ*relP*::NPKm^r	22
Δ*relRS*	Δ*relRS*::NPKm^r	22
Δ*relAPQ*	Δ*relA* Δ*relP* Δ*relQ*::Erm^r NPKm^r Tet^r	22
UA159::P_{835}-*cat*	Sp^r Km^r	This study
Δ*relP*::P_{835}-*cat*	Sp^r Km^r	This study
UA159::P_{relP}-*cat*	Sp^r Km^r	This study
Δ*836p*::P_{relP}-*cat*	Sp^r Km^r	This study
Plasmids		
pJL105	CAT fusion integration vector based on pJL84,[a] Sp^r Km^r	This study
pDL278	*E. coli-Streptococcus* shuttle vector, Sp^r	5
pMSP3535	*E. coli-Streptococcus* shuttle vector, Erm^r	9

[a] pJL84 is described elsewhere (47).

mechanisms for modulating (p)ppGpp levels under nonstringent conditions (22).

Of particular interest, the *relP* gene is part of an operon encoding a two-component signal transduction system (TCS) (RelRS), and the RelP enzyme appears to produce the bulk of (p)ppGpp that is present during exponential growth (22). The RelRS TCS is required for optimal expression of *relP*, suggesting that *S. mutans* has the ability to modulate (p)ppGpp levels by sensing some as-yet-undefined extracellular stimulus. Interestingly, inactivation of *relRS* leads to an increased growth rate of the organism and higher final yields in batch culture (22). Also, mutants deficient in RelP had aberrant stress responses when they were cultivated in biofilms (22). Thus, RelP affects the growth and homeostasis of *S. mutans*, perhaps through a quorum-sensing pathway. In contrast, the contribution of RelQ to (p)ppGpp production was minimal under normal growth conditions and following treatment of the cells with mupirocin or serine hydroxymate (22). Presently, the role of RelQ in (p)ppGpp metabolism and cellular homeostasis is under investigation in our laboratory.

S. mutans is a facultative anaerobe and can metabolize oxygen, but it is catalase negative, lacks a complete tricarboxylic acid (TCA) cycle and respiratory chain, and has a limited capacity to metabolize reactive oxygen species. Growth and especially biofilm formation by *S. mutans* are impaired in the presence of oxygen (1, 2). Comparisons of the transcriptional profiles of *S. mutans* growing with aeration versus under anaerobic conditions revealed that many genes are regulated by oxygen or redox potential, including *relP* (4) and the *SMu0835* to *SMu0839* gene cluster (Oralgen database; http://www.oralgen.lanl.gov; GenBank locus tag *SMU.921* to *SMU.925*, respectively) located immediately upstream of *relP*. In addition, our lab reported that the uncharacterized *SMu0835*, *SMu0836*, and *SMu0837* genes were upregulated in response to mupirocin treatment in a (p)ppGpp-dependent manner (35), identifying a possible connection of this gene cluster to (p)ppGpp and stress tolerance. Given the importance of (p)ppGpp metabolism in the regulation of homeostasis and virulence pathways in *S. mutans* and the potential regulatory overlap of *relP* and *SMu0835* to *SMu0837*, we characterized a variety of mutants lacking some or all of these genes. The results reveal that the gene products play essential roles in physiologic homeostasis by linking growth regulation, stress tolerance, biofilm formation, and genetic competence, all of which are traits that are crucial for persistence and virulence of this important human pathogen.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Escherichia coli* strains were grown in Luria broth supplemented with chloramphenicol (20 μg ml^{-1}) or ampicillin (100 μg ml^{-1}), when necessary. *S. mutans* UA159 and its derivatives were maintained in brain heart infusion (BHI) medium (Difco) supplemented with kanamycin (1 mg ml^{-1}) or spectinomycin (1 mg ml^{-1}), when necessary. For biofilm assays, a semidefined biofilm medium (BM) (27) supplemented with 10 mM sucrose or 20 mM glucose was used. For growth studies, cultures were grown overnight in BHI broth at 37°C in a 5% CO_2 atmosphere and then diluted 1:50 into fresh BHI broth and grown to mid-exponential phase (optical density at 600 nm [OD_{600}] = 0.5). The cultures were then diluted 1:100 in 400 μl of BHI broth in multiwell plates, overlaid with sterile mineral oil, and placed in a Bioscreen C growth monitor at 37°C. The optical density (OD_{600}) was measured every 30 min for 24 h with shaking for 15 s before each reading.

DNA manipulation and construction of mutants and reporter gene fusions. A series of mutant strains (Table 1) were derived from *S. mutans* UA159 using PCR ligation mutagenesis procedures with the insertion of either a polar (ΩKm) or a

nonpolar (NPKm) kanamycin resistance cassette to replace the genes (5). Briefly, primers were used to amplify the regions flanking the genes of interest, the PCR products were digested and ligated to the desired antibiotic resistance cassette, and the mixture was transformed into competent *S. mutans* cells. Transformants were selected on BHI agar with kanamycin. PCR and DNA sequencing were used to ensure that the correct mutation had been introduced and that no mutations were created in the genes immediately upstream or downstream of the insertion site during recombination. The promoter regions of the *SMu0835* and *relP* genes were amplified and cloned into the pJL105 integration vector, which has a staphylococcal chloramphenicol acetyltransferase (CAT) gene (*cat*) that lacks a promoter and ribosome binding site (RBS) (47). The pJL105 vector has sequence homology to the *mtlA* and *phnA* genes, which facilitates double-crossover recombination and integration of the inserted DNA in a single copy in the *S. mutans* chromosome. The *cat*-promoter fusions were transformed into wild-type and mutant strains of *S. mutans*, and the integrity of the strains was verified by PCR and DNA sequencing.

Stress tolerance assays. To assess the ability of the mutants to grow at low pH, cells were grown overnight in BHI broth, diluted 1:100 into fresh BHI, and grown to mid-exponential phase at 37°C in a 5% CO_2 atmosphere. The cells were then diluted 1:100 in BHI broth that was acidified to pH 5.5 with HCl, 400 μl of each sample was added to multiwell plates, 3 drops of sterile mineral oil was added to each well, and growth was monitored in a Bioscreen C growth monitor. When effects of growth in air were assessed, no mineral oil overlay was utilized. For analysis of oxidative stress tolerance, the cells were grown to an OD_{600} of 0.5 in BHI broth at 37°C in a 5% CO_2 atmosphere. The cultures were then diluted 1:100 in BHI broth containing 25 mM paraquat (methyl viologen; Sigma), each well was overlaid with sterile mineral oil, and growth was monitored in a Bioscreen C growth monitor.

Biofilm assays. *S. mutans* strains were grown to mid-exponential phase in BHI broth at 37°C in a 5% CO_2 atmosphere. The cells were diluted 1:100 in BM supplemented with either 20 mM glucose or 10 mM sucrose and transferred to polystyrene microtiter plates. The cells were incubated in a 5% CO_2 atmosphere at 37°C for 24 or 48 h. The medium was decanted and the plates were washed twice with 200 μl of sterile water to remove planktonic and loosely bound cells. The adherent bacteria were stained with 60 μl of 0.1% crystal violet for 15 min. After the bacteria were rinsed twice with 200 μl of water, the bound dye was extracted from the stained biofilm using 200 μl of ethanol-acetone (8:2). Biofilm formation was quantified by measuring the absorbance of the solution at 575 nm.

Transformation assays. Overnight cultures were diluted 1:20 in 200 μl of BHI broth in polystyrene microtiter plates. The cells were grown to an OD_{600} of 0.15 in a 5% CO_2 atmosphere. When desired, 5 μl of synthetic competence-stimulating peptide (CSP; 1 μmol ml^{-1}) (5) was added, cells were incubated for 10 min, and 0.5 μg of purified plasmid pDL278, which harbors a spectinomycin resistance (Sp^r) gene, was added to the culture. After 2.5 h of incubation at 37°C, transformants and total CFU were enumerated by plating appropriate dilutions on BHI agar plates with and without 1 mg ml^{-1} spectinomycin, respectively. The numbers of CFU were counted after 48 h of incubation, and transformation efficiency was expressed as the percentage of transformants among the total viable cells.

RNA extraction and quantitative reverse transcriptase PCR (qRT-PCR). Three colonies from each strain were grown overnight in BHI broth, diluted 1:50 in fresh BHI, grown to an OD_{600} of 0.5, and harvested. Total RNA was extracted (1), treated with DNase I, and further purified with an RNeasy minikit (Qiagen). The RNA concentration was measured in triplicate using a spectrophotometer. The purified RNA (1 μg) was used to generate cDNA from gene-specific primers according to the Superscript III first-strand synthesis (Invitrogen) reverse transcription protocol. The gene-specific primers were designed with Beacon Designer (version 4.0) software, and standard curves were prepared for each gene. Real-time PCRs were carried out using an iCyclerQ real-time PCR detection system (Bio-Rad) and iQSYBR green supermix (Bio-Rad) according to the protocol provided by the supplier. Triplicates of each cDNA sample along with cDNA controls for each of the triplicate isolates analyzed were subjected to real-time PCR. Statistical analysis of the data was performed as detailed elsewhere (3).

Chloramphenicol acetyltransferase assay. Strains carrying promoter-*cat* gene fusions were grown in 50 ml of BHI broth in a 5% CO_2 atmosphere at 37°C to an OD_{600} of 0.5. Protein was extracted by bead beating in the presence of glass beads for 20 s twice with a 2-min interval on ice. The cell lysates were centrifuged at 4,000 × *g* for 10 min, and the supernatants were used for measuring CAT activity by the method of Shaw et al. (44). The concentration of protein was measured using the bicinchoninic acid assay (Thermo Scientific). CAT activity was expressed as nmol of chloramphenicol acetylated min^{-1} (mg protein)$^{-1}$.

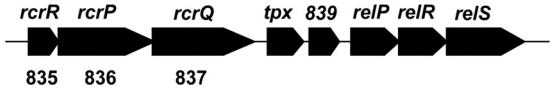

FIG. 1. Schematic diagram of the *SMu0835-SMu0836-SMu0837-SMu0838-SMu0839* (*rcrRPQ*) gene cluster and the *relPRS* operon in *S. mutans* UA159. *SMu0835* (*rcrR*) encodes a predicted transcriptional regulator of the MarR family, *SMu0836* (*rcrP*) and *SMu0837* (*rcrQ*) are annotated as an ABC-type multidrug/protein/lipid transport system, *SMu0838* encodes a thiol peroxidase, *SMu0839* encodes a predicted bacteriocin immunity protein, *SMu0840* (*relP*) encodes a GTP pyrophosphokinase, *SMu0841* (*relR*) encodes a response regulator, and *SMu0842* (*relS*) encodes a sensor histidine kinase of a classic two-component system.

Measurement of (p)ppGpp accumulation. Accumulation of (p)ppGpp in *S. mutans* was done as described elsewhere (22). Briefly, cells from an overnight culture were diluted 1:25 into the chemically defined medium FMC to an OD_{600} of 0.2. Cells were labeled with [^{32}P]orthophosphate for 1 h at 37°C and harvested, and (p)ppGpp was extracted with formic acid (22). If desired, 0.003% hydrogen peroxide was added to the samples at the same time as the label. Following the extraction, the numbers of cpm/μl of the supernatant fraction was measured in a scintillation counter, and 2.0×10^5 cpm of each sample was spotted onto a polyethyleneimine (PEI) cellulose plate (Selecto Scientific) for thin-layer chromatography (TLC). Solutes were resolved and detected using the buffers and conditions detailed elsewhere (22). The accumulation of ppGpp (GP4) and pppGpp (GP5) was quantified using an AlphaEase FC (Fluorochem 8900) imaging system spot density analysis tool. The density of the spots was quantified as integrated density value (IDV) per area.

RESULTS

Organization of *SMu0835-SMu0836-SMu0837-SMu0838-SMu0839* gene cluster. Figure 1 shows a schematic of the *SMu0835*, *SMu0836*, *SMu0837*, *SMu0838*, and *SMu0839* genes (GenBank accession numbers *SMU.921* to *SMU.925*, respectively) and *relPRS* genes. *SMu0835*, which we designated *rcrR* for r*el c*ompetence-*r*elated regulator, is predicted to encode a cytoplasmic transcriptional regulator with a winged helix-turn-helix DNA binding motif and is identified (Oralgen, Los Alamos, NM) to be a member of the *m*ultiple *a*ntibiotic *r*esistance (MarR) family of transcriptional regulators (6). MarR-type regulators are widely distributed in bacteria and regulate many functions, including resistance to xenobiotics and oxidative stressors and expression of virulence genes (14). For example, the *E. coli* MarR protein modulates the expression of multiple genes that impact resistance to a variety of antibiotics and to oxidative stress (7, 40). *SMu0836* (*rcrP*) and *SMu0837* (*rcrQ*) are apparently cotranscribed with *SMu0835* and are annotated (Oralgen) as transmembrane ATP-binding cassette (ABC) transporters that function as multidrug/protein/lipid transport systems. In both gene products, the ATP-binding cassette and transmembrane domains are located on a single polypeptide and are classified as type 6 transporters (13), which include homo- and heteromeric ABC transporters that typically function as exporters involved in the externalization of toxic compounds or peptides. The highly conserved phosphate-binding Walker A sequence and Walker B magnesium-binding sequences, as well as the signature LSGGQ sequence, found in ABC transporters are conserved in both open reading frames. Notably, we could identify MarR-like regulators that are linked to two similar ABC transporters in multiple other streptococcal species and in other members of the phylum *Firmicutes* (see Table S2 in the supplemental material).

TABLE 2. Summary showing mutant strain *SMu0835*, *SMu0836*, and *SMu0837* mRNA expression levels and transformation efficiency compared to those for the wild-type strain

Strain	Expression			Transformation efficiency[a]	
	SMu0835	*SMu0836*	*SMu0837*	+CSP	−CSP
Δ*835np*	Deleted	100-fold increase	100-fold increase	None	None
Δ*835p*	Deleted	Wild-type levels	Wild-type levels	10-fold increase	10^4-fold increase
Δ*835-837np*	Deleted	Deleted	Deleted	None	None
Δ*836p*	Wild-type levels	Deleted	1,000-fold decrease	10-fold decrease	100-fold increase
Δ*836-837np*	Wild-type levels	Deleted	Deleted	10-fold decrease	100-fold increase
835$^+$/Δ*835np*	Wild-type levels	Wild-type levels	Wild-type levels	Wild-type levels	Wild-type levels
835$^+$/Δ*835p*	Wild-type levels	Wild-type levels	Wild-type levels	Wild-type levels	Wild-type levels
835$^+$/Δ*835-837np*	10-fold increase	Deleted	Deleted	None	None

[a] CSP, exogenous CSP added; −CSP, no CSP added; none, zero transformants were detected when 0.04 ml of the transformation mixture was plated directly onto selective medium.

SMu0838 (*tpx*) encodes a thiol peroxidase that was shown by microarray analysis to be upregulated in response to growth with aeration compared to anaerobically growing cells (4). Thiol peroxidases can protect bacteria against oxidative stress by breaking down hydrogen peroxide and organic hydroperoxides. The Tpx protein contains the 2 cysteine residues that form the conserved CXXC motif and that are oxidized by hydroperoxides to form a sulfenic acid. Notably, there are other proteins in *S. mutans* that contain the FX4 CXXC motif that are involved in responses to aeration, including SMu0629, which modulates the activity of the AtlA autolysin (4). *SMu0839* (*cipI*) was shown by Levesque and coworkers (38) to encode a bacteriocin immunity protein. Bacteriocin immunity proteins are generally integral membrane proteins that confer protection against certain classes of antimicrobial agents and often enhance stress tolerance (31). In *S. mutans*, CipI plays a protective role against the CipB bacteriocin-like protein, which appears to function intracellularly and which contributes to cell death following exposure to high levels of CSP (38).

Transcriptional organization of *SMu0835-SMu0836-SMu0837* operon. A collection of mutants was made by allelic exchange using either polar or nonpolar antibiotic resistance cassettes (Table 1). Central to the interpretation of the results obtained with these mutants is that the expression level of the ABC transport genes (*SMu0836* and *SMu0837*) has a profound impact on the phenotype of the mutants, particularly genetic transformation. Thus, Table 2 is included to summarize the salient properties of the various mutant strains and the measured effects of each mutation on downstream gene expression and transformation efficiency of each strain in the presence or absence of exogenous competence-stimulating peptide. RT-PCR and quantitative real-time RT-PCR were used to determine whether genes could be cotranscribed and to measure stable transcript levels of the genes in the *SMu0835*, *SMu0836*, *SMu0837*, *SMu0838*, and *SMu0839* operon in the Δ*835np*, Δ*836np*, Δ*836p*, and Δ*837np* mutants. RT-PCR revealed that *SMu0835* can be cotranscribed with *SMu0836* and *SMu0837* as a polycistronic operon (data not shown).

Consistent with the RT-PCR results, the expression of *SMu0837* was downregulated nearly 1,000-fold in the polar Δ*836p* mutant compared to the level of expression in the parental strain (Table 2; Fig. 2B). In contrast, expression of *SMu0838* (*tpx*) or *SMu0839* (*cipI*) was not significantly altered in the Δ*836p* mutant compared to that in the wild-type strain (data not shown). The lack of influence of polar insertions in *SMu0836-SMu0837* on *tpx* mRNA levels was consistent with our inability to detect a product in RT-PCRs using primers that would amplify across the *SMu0837-tpx* intergenic region (data not shown), indicating that these genes are not part of the *SMu0835-SMu0836-SMu0837* operon.

Interestingly, the expression of *SMu0836-SMu0837* was significantly upregulated (~100-fold) in the mutant carrying a nonpolar insertion in the *SMu0835* gene (Δ*835np*) compared to the level of expression in the wild-type strain (Fig. 2A and B; Table 2), adding further support to the suggestion that these three genes constitute an operon and revealing that the MarR-like protein may repress expression of this operon. To further demonstrate that *SMu0835* was autogenously regulated, we measured the levels of the mRNA for the nonpolar kanamycin resistance gene that was used to replace *SMu0835* in the Δ*835np* strain, which lacks the MarR-like regulator, and compared it to the expression levels in the Δ*836np* mutant, which has an intact MarR-like regulator. This nonpolar kanamycin resistance cassette lacks a promoter (5), so its expression was driven entirely by the *SMu0835* promoter in both the Δ*835np* and Δ*836np* strains. Consistent with the real-time PCR results, the expression of the nonpolar kanamycin marker was about 100-fold higher in the Δ*835np* mutant than in the Δ*836np* mutant (Fig. 2C), adding additional support to the hypothesis that the SMu0835 protein is a negative regulator of the *SMu0835-SMu0836-SMu0837* operon. Also of interest is that the magnitude of induction of the operon associated with loss of the SMu0835 protein indicates that the genes are significantly repressed during exponential growth in rich medium. Notably, though, the expression of this operon was not subject in any significant way to growth phase control (data not shown).

Growth characteristics of mutants. The growth of the various mutants (Table 1) in BHI broth was monitored. All of the mutants constructed using the nonpolar cassette displayed a modestly extended lag phase and a slightly lower growth rate than the wild-type strain. The wild-type strain had a doubling time of about 46 min ± 1.5 min, whereas the nonpolar mutants had a doubling time of about 51 min ± 3.5 min (Fig. 3A). However, the final optical density attained by the nonpolar mutants was consistently lower than that attained by the parental strain. Notably, the Δ*836p* polar mutant, which would not express either ABC transporter, exhibited much slower

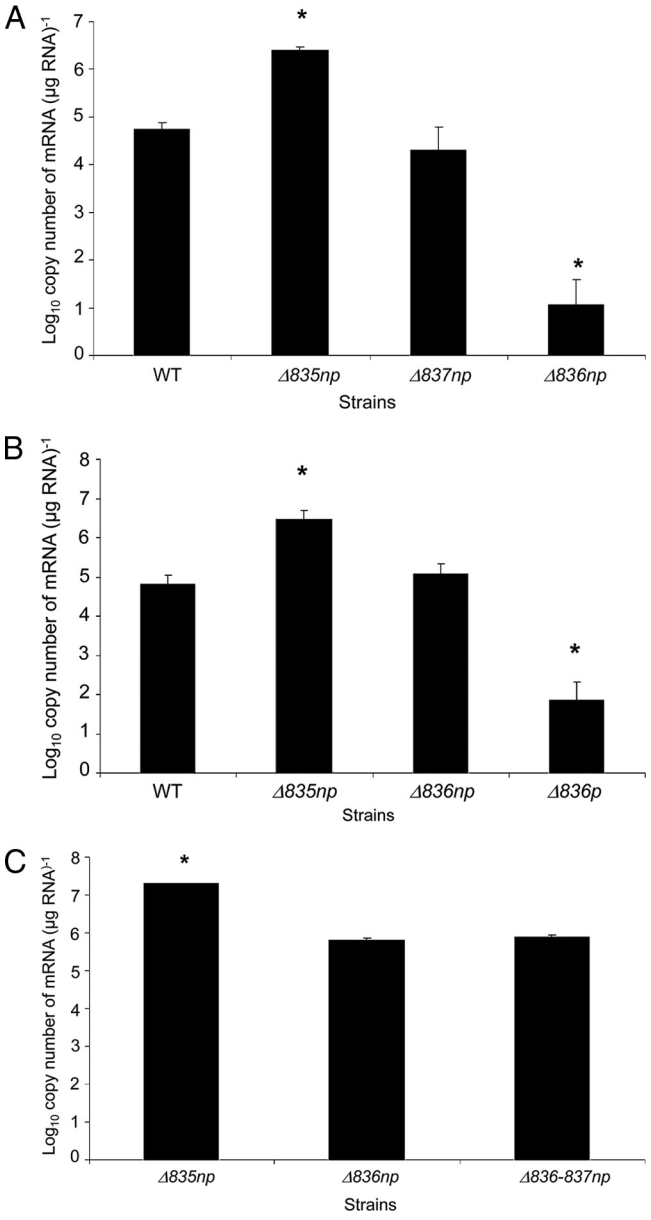

FIG. 2. Real-time RT-PCR. *SMu0836* (A), *SMu0837* (B), and nonpolar kanamycin resistance cassette (C) mRNA levels are shown. In all cases, cells were grown to mid-exponential phase (OD_{600} = 0.5), total RNA was extracted, and reverse transcription was done using gene-specific primers followed by quantitative real-time PCR. The data are presented as the copy number of each gene per μg of input RNA. *, differs from the wild type at $P < 0.05$ (Student's *t* test). WT, wild type.

growth (85 min ± 7 min) and lower final yields than the nonpolar mutants or parental strain (Fig. 3B). Both the polar and nonpolar *tpx* mutants had growth rates similar to those for the nonpolar mutants (data not shown), so the effects of the loss of the ABC transporters were independent of the level of expression of *tpx* or *cipI*.

To test if the mutants were sensitive to low pH, the strains were grown in BHI broth that was acidified to pH 5.5 with HCl (Fig. 3C). All of the mutants had a slower growth phenotype than the wild-type strain. Importantly, the Δ*836polar* mutant grew very poorly at pH 5.5 and showed very little cell accumulation even after 26 h (Fig. 3D). Exposure to oxygen has been shown to alter growth and biofilm formation by *S. mutans* (1), and some of the genes in the *SMu0835-SMu0836-SMu0837-SMu0838-SMu0839* gene cluster were upregulated in response to oxygen (4). The growth phenotype of the mutants was assessed in BHI without an oil overlay, thus exposing them to air during growth, or in medium containing 25 mM paraquat, a superoxide-generating agent, with an oil overlay (Fig. 4). Cells could not grow in the presence of paraquat without an oil overlay. The Δ*836p* polar mutant again had a lower growth rate than the other strains. In addition, the Δ*836np* and Δ*837np* mutants displayed a slow-growth phenotype when the cells were exposed to air (Fig. 4A). Interestingly, the Δ*relP* mutant exhibited significantly faster growth and a shorter lag phase than the wild-type strain in medium containing paraquat or in cultures that were grown with exposure to air (Fig. 4B).

S. mutans has the ability to form biofilms, an essential process in establishment, persistence, and pathogenesis. Efflux pumps have been shown to be required for biofilm formation in certain organisms, and compounds that can inhibit efflux pumps can affect the ability of bacteria to form biofilms (18). We evaluated whether the various mutants had impaired biofilm formation in microtiter plates in BM broth containing sucrose or glucose. Biofilm formation was quantified at 24 h and 48 h to take into account the effects of the slower-growth phenotypes of some of the mutants. There were no significant differences in biofilm formation seen after 24 h or 48 h when the strains were grown in sucrose. However, when the strains were grown in glucose, the Δ*835np* mutant, which overexpresses the ABC exporters (Table 2), and the Δ*836p* mutant, which lacks both ABC transporters, formed less biofilm than the other strains at both time points (Fig. 5). Collectively, these data highlight the requirement of the ABC transporters for stress tolerance and biofilm formation, but they also show that loss of both transporters is required to observe changes in the phenotypes of interest.

Competence defect in mutants. *S. mutans* is naturally competent, and elements of the competence regulon have been linked to stress tolerance and biofilm formation (23, 24). The transformation efficiency of the various mutant strains was assessed with and without the addition of synthetic CSP. The transformation efficiency of the mutants was strongly dependent on the expression of *SMu0835* and on the expression levels of the *SMu0836* and *SMu0837* ABC exporters (Table 2). Specifically, compared with the wild-type strain, the Δ*836p* mutant, lacking both ABC exporters, had a lower transformation efficiency than the parental strain when CSP was provided (~10-fold decrease) but a higher transformation efficiency than the parental strain in the absence of CSP (~100-fold increase) (Tables 2 and 3). Of particular interest, we were unable to obtain even a single transformant of the Δ*835np* strain, which lacks the MarR-like regulator and overexpresses the ABC porter genes, or the strain lacking all three genes (strain Δ*835-837np*), regardless of whether exogenous CSP was added (Tables 2 and 3). We also attempted to transform the Δ*835np* and Δ*835-837np* mutants with plasmid pMSP3535 (9), which, like pDL278, is a shuttle vector, but it carries an erythromycin resistance marker instead of a spectinomycin resistance marker, as well as with the pJL105 integration vector

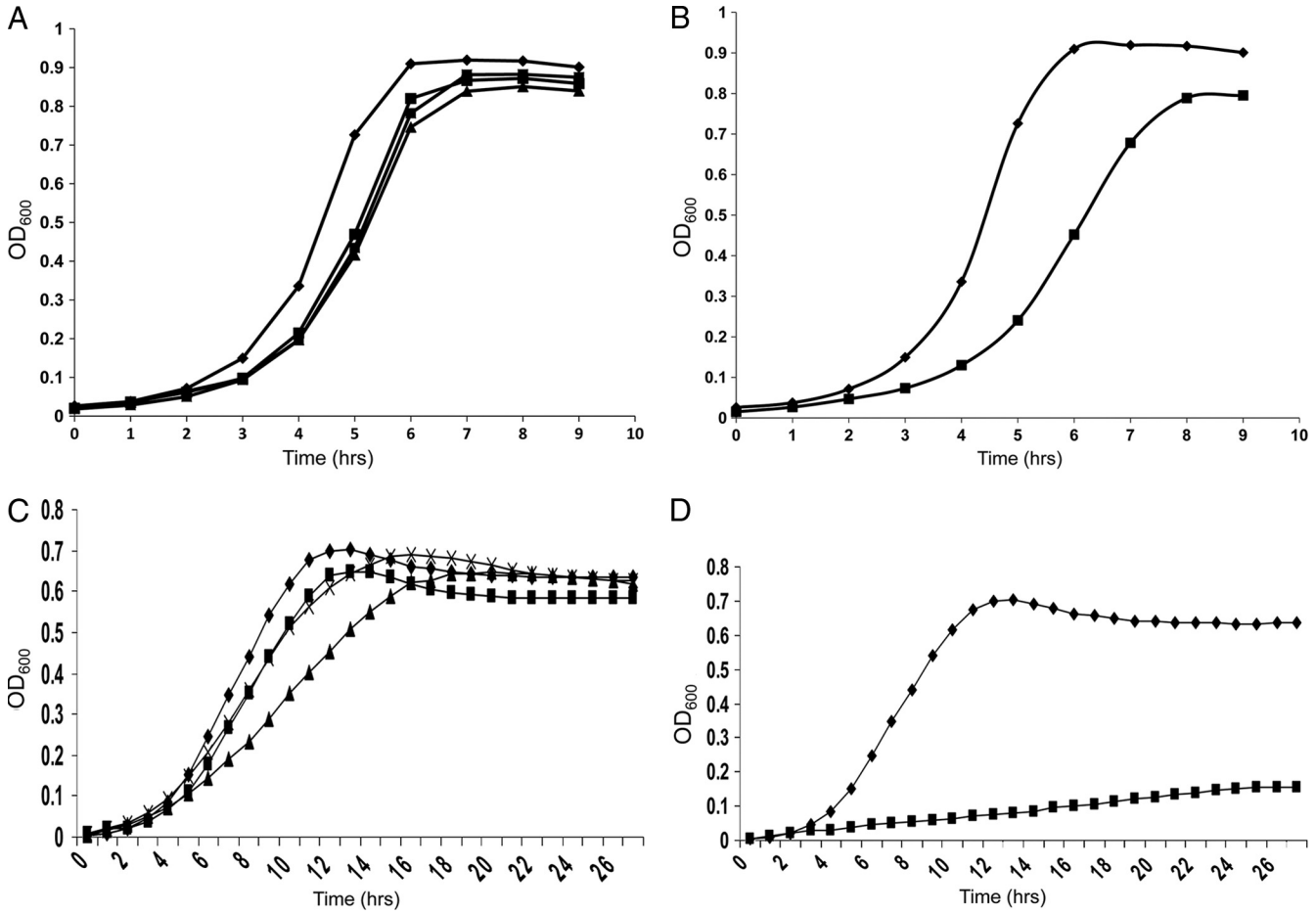

FIG. 3. Comparison of wild-type and mutant strain growth. (A) Growth of nonpolar strains versus that of the wild type. The strains were grown in triplicate to mid-exponential phase in BHI broth, diluted 1:100, transferred to fresh BHI broth, overlaid with sterile mineral oil, and placed in a Bioscreen C apparatus at 37°C to monitor growth. Diamonds, wild type; squares, Δ*835np*; ×, Δ*837np*; triangles, Δ*836np*. (B) Growth of the Δ*836p* versus the wild type. Strains were grown as described for panel A. Diamonds, wild type; squares, Δ*836p*. (C) Growth of the nonpolar strains versus that of the wild type at pH 5.5. Cells were grown to mid-exponential phase in BHI broth, diluted 1:100 in BHI broth acidified to pH 5.5, covered with sterile mineral oil, and placed in a Bioscreen C apparatus at 37°C to monitor growth. Diamonds, wild type; squares, Δ*835np*; ×, Δ*837np*; triangles, Δ*836np*. (D) Growth of Δ*836p* versus the wild type at pH 5.5. Strains were grown as described for panel C. Diamonds, wild type; squares, Δ*836p*. The results are representative of those from three independent experiments performed in triplicate.

(Table 1). In neither case was any transformant isolable (data not shown).

Interestingly, we also analyzed a mutant in which the ΩKm marker (5) was used to replace the *SMu0835* gene (Δ*835polar*). qRT-PCR analysis of the Δ*835p* polar mutant revealed that *SMu0836* and *SMu0837* were expressed at levels similar to those for the wild-type strain (Table 2; see Fig. S1B in the supplemental material). We determined that expression of the genes downstream of the polar marker was not due to an internal promoter in *SMu0835*, since cloning of the 3′ portion of this gene behind a reporter gene gave no detectable expression in *S. mutans* (data not shown). Instead, since loss of *SMu0835* caused roughly a 100-fold increase in the transcription of *SMu0836-SMu0837* (Table 2; Fig. 2), we believe that high-level activation of the *SMu0835* promoter in the strain lacking the MarR-like regulator leads to enhanced read-through of the terminator in the ΩKm element. Thus, if the ΩKm terminator was roughly 99% efficient, then the observed results would be expected. Importantly, the Δ*835polar* strain, lacking the MarR regulator but expressing the exporters at levels that were essentially the same as those for the wild-type strain (Table 2; see Fig. S1B in the supplemental material), was hypertransformable. In particular, even in the absence of exogenously added synthetic CSP (Table 3), nearly 10^4-fold more transformants were obtained with the Δ*835polar* strain than with the wild-type strain.

To determine if the results were due to loss of the MarR-like transcriptional repressor, as well as to changes in the transcription of the ABC porter genes, we introduced a wild-type copy of the *SMu0835* gene expressed from its own promoter on the shuttle plasmid pDL278 into the Δ*835p*, Δ*835np*, and Δ*835-837np* mutant strains (Table 1; see Fig. S1 in the supplemental material). Complementation of the Δ*835np* and Δ*835p* mutants, which resulted in strains 835^+/Δ*835np* and 835^+/Δ*835p*, respectively, restored the wild-type transformation efficiency and wild-type levels of *SMu0836* gene expression (Table 2; see Fig. S1 and S4 in the supplemental material). However, introduction of the MarR-like regulator into the strain lacking all

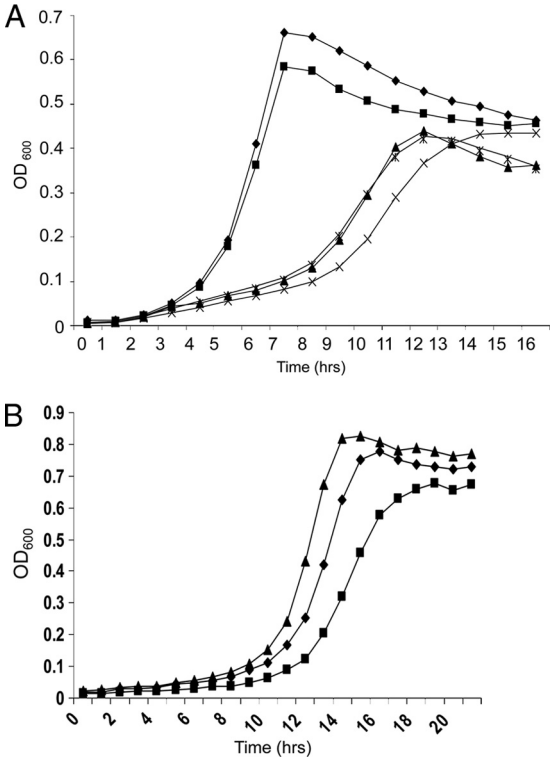

FIG. 4. Growth with oxidative stress. (A) Growth of mutant strains versus wild-type strain without oil overlay. The different strains were grown to mid-exponential phase in BHI broth and then diluted 1:100 into fresh BHI broth and placed in a Bioscreen C apparatus at 37°C to monitor growth. Diamonds, wild type; squares, Δ*835np*; triangles, Δ*836np*; asterisks, Δ*837np*; ×, Δ*836p*. (B) Growth of the mutants versus the wild-type strain in 25 mM paraquat. Cells were grown to mid-exponential phase in BHI broth and then diluted 1:100 in BHI that contained 25 mM paraquat. Diamonds, wild type; squares, Δ*836p*; triangles, Δ*relP*. The results are representative of those from three independent experiments performed at least in triplicate.

three genes, strain *835*$^+$/Δ*835-837np*, which still lacked the SMu0836 and SMu0837 transporters, did not restore transformation (Table 2; see Fig. S4 in the supplemental material). Clearly, then, the absolute expression levels of the ABC transporter genes, as well as the presence of the MarR-like regulator, are critical for competence development. Since it is not possible to control the level of expression of complementing genes in *S. mutans* with any precision, we did not complement the mutant strains with the ABC transport genes. Thus, a more reliable picture of the contribution of these gene products to stress tolerance and their impact on competence was garnered by contrasting the behaviors of the various polar and nonpolar mutants and complemented strains. Once the substrate(s) for the porters is identified, efficiency of translocation of the substrate(s) by particular mutants can be correlated with phenotype.

***SMu0835*, *SMu0836*, and *SMu0837* affect *comX* and *comY* expression.** To begin to understand the basis for the changes in the transformation efficiency in strains with aberrant expression of the gene for the MarR-like regulator and ABC transporters, we measured the levels of expression of *comD*, *comX*, and *comYA* in the different mutant strains by qRT-PCR (5, 32).

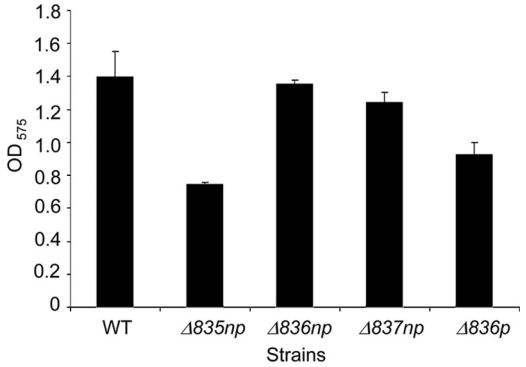

FIG. 5. Biofilm formation. The differences in biofilm formation of the mutants compared with that of the wild-type (WT) strain in glucose. Cells were grown to an OD_{600} of 0.5 in BHI broth and then diluted 1:100 into BM supplemented with 20 mM glucose in microtiter plates. Biofilm formation was quantified after 48 h, as described in Materials and Methods. The results are representative of those from three independent experiments performed at least in triplicate.

ComD is the response regulator in the two-component system involved in sensing of CSP, *comX* encodes an alternative sigma factor that is under the control of ComD, and *comYA* is required for competence development and is transcriptionally activated by ComX. The qRT-PCR analysis showed that the expression of *comD* was not affected in the mutant strains compared to the effect in the wild-type strain (data not shown). In contrast, the expression of *comYA* was downregulated nearly 15-fold in the nontransformable Δ*835np* and Δ*835-837np* mutants and was upregulated by more than 100-fold in the hypertransformable Δ*835p* polar strain (Fig. 6A). Interestingly, the expression of *comX* was upregulated nearly 100-fold in both the Δ*835np* and Δ*835p* mutant strains (Fig. 6B; see Fig. S2 and S3 in the supplemental material), but the effect on *comY* expression in these strains was markedly different. Therefore, the effects of mutations in or changes in the expression levels of *SMu0835*, *SMu0836*, and *SMu0837* may affect competence by interfering with ComX activation of *comY*. Also relevant is that complementation analysis revealed that the *835*$^+$/Δ*835np* and *835*$^+$/Δ*835p* strains expressed wild-type levels of *comX* and *comYA* (see Fig. S2 and S3 in the supplemental material).

***SMu0835*, *SMu0835*, and *SMu0837* influence *relP* expression and vice versa.** Our laboratory previously reported that the

TABLE 3. Transformation efficiencies of wild-type and various mutants strains in the presence or absence of added CSP

Strain	% Transformants[a]	
	+CSP	−CSP
UA159	1.7×10^{-3}	1.44×10^{-6}
Δ*835np*	0	0
Δ*835p*	4.0×10^{-2}	1.0×10^{-2}
Δ*835-837np*	0	0
Δ*836p*	1.87×10^{-4}	2.39×10^{-4}
Δ*836-837np*	1.61×10^{-4}	1.24×10^{-4}
Δ*tpx np*	3.25×10^{-3}	2.26×10^{-6}

[a] Percent transformants = (number of transformants/total number of viable bacteria) × 100. +CSP, exogenous CSP added; −CSP, no CSP added.

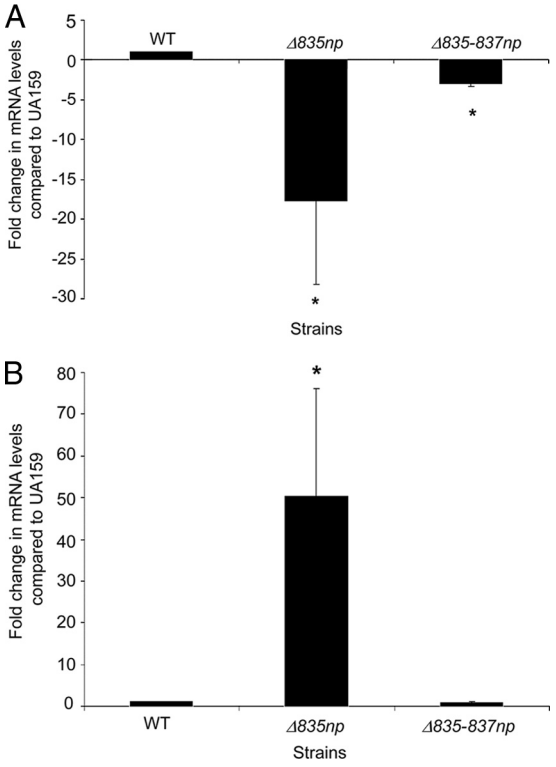

FIG. 6. Quantitative real-time PCR. *comYA* (A) and *comX* (B) mRNA levels are shown. In both cases, cells were grown to mid-exponential phase (OD_{600} = 0.5), total RNA was extracted, and reverse transcription was done using gene-specific primers, followed by quantitative real-time PCR. The copy number of each mRNA per μg of input RNA was quantified. The data represent the difference in fold change in copy number of the mutant strains compared to the copy number for the wild-type (WT) strain, where the wild-type level was set at 1.0. *, differs from the wild type at $P < 0.05$ (Student's *t* test).

expression of *tpx*, *SMu0839*, and *relP* was enhanced in cells growing with aeration (4). Interestingly, members of our group also showed that the *SMu0835-SMu0836-SMu0837* operon was upregulated during a mupirocin-induced stringent response in a (p)ppGpp-dependent manner (35), i.e., with no induction of these genes in a *relA* mutant. Therefore, the expression of the *relP* and *SMu0836-SMu0837* operons may be coordinated with one another in response to particular stresses or signals. qRT-PCR was performed to compare the amount of mRNA expressed from the *relPRS* operon in the wild-type strain with that expressed in the various mutant strains. The expression level of the *relP* operon in the Δ*835np* and Δ*836p* mutants, which either overexpress or do not express the exporters, respectively, was approximately half that measured in the wild-type strain, whereas the expression level of *relP* and *relRS* in the Δ*837np* and Δ*839* mutants was similar to that in the wild-type strain (Fig. 7A and B). Similarly, qRT-PCR analysis was done to measure the expression of *SMu0835* and *SMu0836* in a *relP* mutant strain, and it was noted that the expression of these genes was approximately 3-fold lower in the strains lacking *relP* or *relRS* than in the wild-type strain (Fig. 7C and D). In contrast, the levels of expression of *SMu0835* and *SMu0836* were similar in a Δ*relA* mutant and the wild-type strain (data not shown), an expected finding, given the knowledge that RelA does not contribute to (p)ppGpp pools under normal growth conditions (22).

To verify the qRT-PCR results, the promoter for *relP* was fused to a promoterless chloramphenicol acetyltransferase (*cat*) gene and CAT activity was measured in exponentially growing cells. Consistent with the qRT-PCR data, *relP* promoter activity was lower in the Δ*836p* mutant than in the wild-type background (Fig. 8A). Using a *cat*-gene fusion as described above, *SMu0835* promoter activity was analyzed in the Δ*relP* genetic background and was found to be 1.5-fold lower than that in the wild-type genetic background (Fig. 8B). Thus, the *cat* fusion data were consistent with the qRT-PCR data showing that expression of the *SMu0835-SMu0836-SMu0837* operon was decreased significantly in the Δ*relP* and Δ*relRS* mutants and vice versa.

RelP is the principal source of (p)ppGpp in exponentially growing cells, and it appears that the RelPRS system functions to slow the growth of the bacteria in response to signals associated with stress, especially oxidative stress (Fig. 4B). To verify that the changes in *relP* expression noted in the *SMu0835-SMu0836-SMu0837* operon mutants were associated with alterations in the levels of (p)ppGpp in cells, alarmone production was monitored in exponentially growing cells in the wild-type strain and selected mutants. The results demonstrated that (p)ppGpp production was adversely affected by loss of constituents encoded by the *SMu0835-SMu0836-SMu0837* operon, consistent with the changes in *relP* mRNA levels and *relP* promoter activity in the same mutants (Fig. 9; Table 4).

DISCUSSION

The ability of *S. mutans* to survive and persist under the continually varying conditions in the oral cavity is intimately associated with its pathogenicity (10), so understanding how the organism adapts to these often hostile conditions can facilitate the development of novel strategies to compromise the persistence or virulence of this important human pathogen. The development of genetic competence, the induction of efflux pumps, and the accumulation of (p)ppGpp are well-documented mechanisms for bacterial adaptations to new environments and to various stressors. The results presented here show that the products of the previously uncharacterized *SMu0835-SMu0836-SMu0837* operon, which we now designate the *rcrRPQ* operon, for r*el-c*ompetence-*r*elated genes, play a major role in the regulation of growth, stress tolerance, and competence development while strongly influencing the expression of the primary (p)ppGpp biosynthetic pathway that is expressed in exponentially growing *S. mutans*. Although *tpx* and *cipI* participate in particular aspects of stress tolerance and the response to CSP, respectively (38), we focused on the *rcrRPQ* operon because deletion of *tpx* or *cipI* had no significant effects on growth, stress tolerance, or gene expression under the conditions that we used for testing. We also found no evidence to support the suggestion that *tpx* or *cipI* was part of the *rcr* operon or had an influence on *relP* expression.

The data presented here demonstrate that the RcrP (SMu0836) and RcrQ (SMu0837) exporters are critical for tolerance of the two environmental stresses that have the greatest influence on the composition (21), biochemical activ-

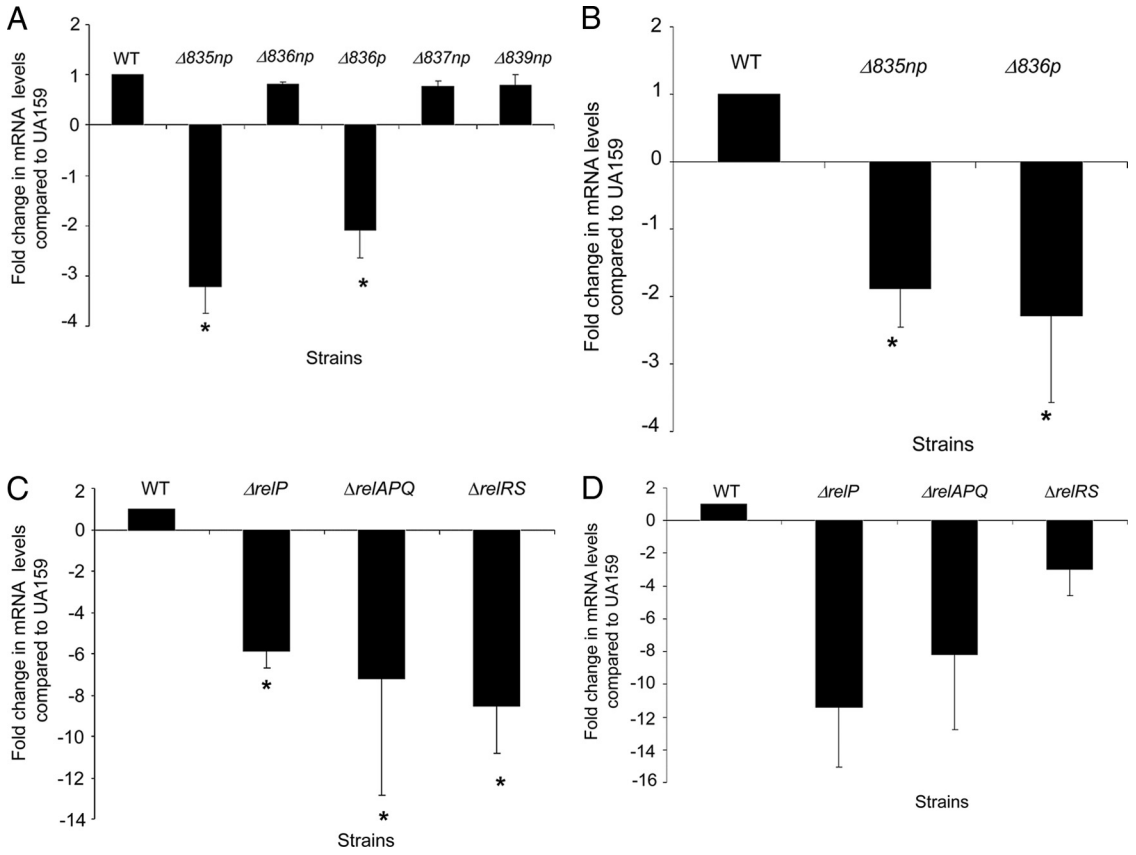

FIG. 7. Quantitative real-time PCR. *relP* (A), *relRS* (B), *SMu0835* (C), and *SMu0836* (D) mRNA levels are shown. In all cases cells were grown to an OD_{600} of 0.5, total RNA was extracted, and reverse transcription was done using gene-specific primers, followed by quantitative real-time PCR. The copy number per μg of input RNA was calculated. The data represent the fold change in copy numbers of the mutant strains compared to the copy number for the wild-type (WT) strain, where the wild-type value was set to 1.0. ∗, differs from the wild type at $P < 0.05$ (Student's *t* test).

ities, and pathogenic potential of oral biofilms: oxidative and acid stress. Strains lacking the RcrPQ transporters grew significantly slower than the parental strain in air and in the presence of the superoxide generator paraquat. Likewise, mutants lacking these transporters were more acid sensitive than the parental strain, as evidenced by slower growth at pH 5.5. The RcrPQ transporters were also necessary for proper biofilm formation, another attribute associated with the pathogenicity of *S. mutans*. While it is not yet established if the RcrP and RcrQ pumps are able to function as a heterodimeric complex, as many ABC porters do (13), RcrP and RcrQ clearly have redundancy in function, since deletion of only one of the exporters caused modest phenotypic changes compared to those seen in strains carrying mutations that affected the expression of both gene products.

ABC efflux transporters play many roles in bacteria and export a wide range of compounds, including antibiotics, metals, peptides, and lipids (29). As noted earlier, RcrPQ have a structure typical of ABC export proteins and are annotated as multidrug/protein/lipid transport systems. The simplest explanation for the growth defects of strains lacking both exporters would be that RcrP and RcrQ are each capable of externalizing a substance or class of compounds that accumulates in growing cells, particularly under aerobic conditions or at low pH. Interestingly, the RcrQ protein is predicted to have a fumarate lyase regulatory domain (Oralgen). Growth of *S. mutans* in air alters metabolism toward heterofermentative growth and impacts the transcriptional profiles of the cells, including enhancing the expression of the genes for pyruvate dehydrogenase and the partial TCA cycle, which in the case of *S. mutans* could lead to enhanced fumarate production (4). We tested whether mutants lacking the ABC transporters were more sensitive to the presence of fumarate, but this was not the case (data now shown). Notably, aeration also increases the production of peptide antibiotics (mutacins) by *S. mutans*, so the transporters could play a role in externalization of peptides with antimicrobial activity. We are currently testing the hypothesis that RcrQP participate in the transport of selected peptide-based moieties. Notwithstanding, multiple findings presented in this communication reveal that the RcrRPQ system in *S. mutans* has key functions in cellular homeostasis, gene regulation, and quorum sensing that extend well beyond simply pumping a deleterious substance from inside the cell.

The overlap between control of expression of the *rcr* operon and the *relP* operon is of considerable interest. The underlying basis for the retention of multiple (p)ppGpp synthases in *S. mutans* and in certain other prokaryotes remains largely enigmatic, but a reasonable explanation for the retention or acqui-

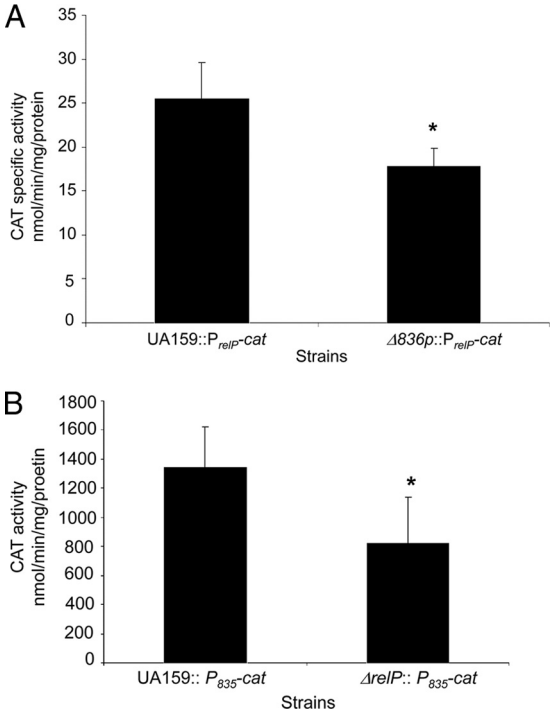

FIG. 8. CAT assays. (A) CAT activity from the *relP* promoter. The *relP* promoter was fused to a promoterless *cat* gene in the pJL105 integration vector. The *cat*-promoter fusion was transformed into the wild-type and Δ*836p* strains. (B) The *SMu0835* promoter was fused to a promoterless *cat* gene in the pJL105 integration vector. The *cat*-promoter fusion was transformed into the wild-type and Δ*relP* mutant strains. In all cases cells were grown to mid-exponential phase (OD_{600} = 0.5) and CAT specific activity was measured as described in Materials and Methods. The results are from three independent experiments performed at least in triplicate. *, differs from the wild type at $P < 0.05$ (Student's *t* test).

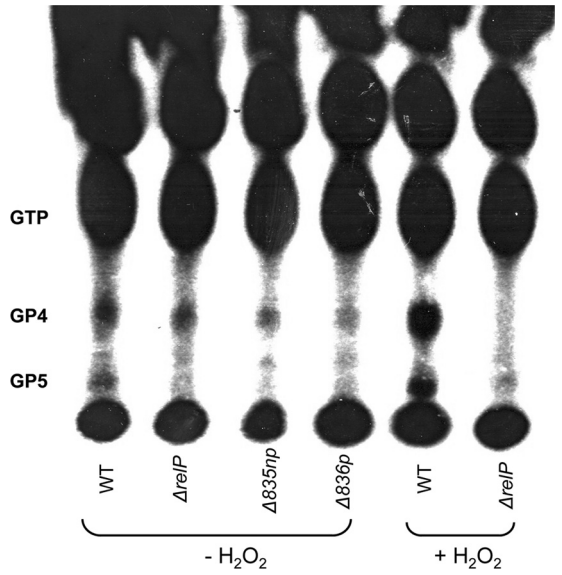

FIG. 9. Accumulation of (p)ppGpp accumulation in mutant versus wild-type (WT) strains with and without 0.003% hydrogen peroxide. Cells were grown to an OD_{600} of 0.2 in FMC and labeled with [^{32}P]orthophosphate. H_2O_2 (0.003%) was added where noted during the labeling. The cells were incubated for 1 h, and (p)ppGpp was extracted using 13 M formic acid. A total of 2×10^5 cpm of each sample was spotted onto PEI-cellulose plates for TLC in 1.5 M KH_2PO_4 buffer.

sition of RelP and the RelRS system during evolution has been disclosed in this study. In particular, the evidence supports the suggestion that the RelRS two-component system may sense oxidative stressors or by-products of oxidative metabolism as a signal to stimulate (p)ppGpp production by activating production of RelP. In previous studies done in our lab, it was shown that the production of (p)ppGpp by RelP plays a significant role in regulating the growth of *S. mutans* in exponentially growing cells (22). It was also shown that overexpression of *relP* in a strain lacking (p)ppGpp hydrolase activity could induce growth arrest (22). Of particular interest here was the substantially higher growth rate of the Δ*relP* mutant strain in the presence of paraquat, likely arising from a failure of the mutant to accumulate (p)ppGpp through RelP activity. We propose that RelP-dependent production of (p)ppGpp in cells exposed to oxidative stress functions to slow the growth of the cells. Since *S. mutans* does not have a complete respiratory chain, has a limited repertoire of enzymes to detoxify reactive oxygen species, and displays much better growth under conditions of lower oxygen tension, RelPRS may help to protect the cells from exhausting limited resources, from overproducing metabolites that are toxic, and from acquiring deleterious mutations.

The signal(s) responsible for activation of the RelRS signal transduction pathway has not yet been identified. In *E. coli*, it has been shown that (p)ppGpp plays a role in resistance to an intrinsic peptide by inducing efflux pumps to extrude the peptide (39). Interestingly, the RcrPQ pumps in *S. mutans* are also induced in response to an increase in (p)ppGpp pools during a mupirocin-induced stringent response (35). Both the *rcrRPQ* and *relPRS* gene products appear to be modulating growth in response to aeration, and on the basis of the results of our CAT assays and real-time PCR analysis, the products of these two operons can exert an effect on the expression of each other. Thus, as part of our working model, we hypothesize that the RcrPQ export apparatus could play a role in externalizing the compound(s) that is detected by RelRS in quorum-sensing fashion to control the growth of *S. mutans* populations in response to elevated redox or reactive oxygen species. Such a feedback loop could at least partially explain the interdependence of *relP* and *rcr* operon expression and the decrease in (p)ppGpp levels in the mutant lacking the RcrPQ porters. This and other potential mechanisms controlling *rcr* and *relP* cross regulation are presently under investigation.

TABLE 4. Percent change in GP4 and GP5 accumulation of mutant strains compared to that of UA159

Strain	% change in IDV compared to that for wild-type strain UA159[a]	
	GP4	GP5
Δ*relP*	10	43
Δ*835np*	26	62
Δ*836p*	30	45

[a] All percentages represent reductions.

RcrR (SMu0835) is the dominant regulator of the *rcrRPQ* operon, repressing the production of the genes for the RcrP and RcrQ transporters under all conditions tested here. Overexpression or uncontrolled expression of efflux pumps can affect bacterial homeostasis and physiology by imposing a metabolic burden on the bacteria or by hypersecreting signaling molecules (25), so it is critical that genes encoding efflux pumps are properly regulated. Of note, the Δ*835np* (*rcrR*-negative) strain had poor biofilm formation in glucose after 48 h, which may be attributable to the gross overexpression of the ABC pumps, although we cannot exclude the possibility that RcrR is influencing the expression of other genes, such as the *com* genes, to affect biofilm formation. We did not find evidence to support the possibility that derepression of the *rcr* operon occurs as a function of growth phase (data not shown), so we propose that accumulation of a specific compound or class of compounds, perhaps those exported by the ABC pumps, could serve as an allosteric regulator of the RcrR transcriptional repressor. Such a model is also supported by the different effects that the various *rcr* mutants have on genetic competence (see below). In addition to the regulation of *rcrQP*, our studies show that RcrR has an influence on the transcription of other genes, including *relPRS*, *comX*, and *comYA*, which links the *rcr* operon to (p)ppGpp production and to development of competence. Whether RcrR exerts its effects directly through binding of the promoter regions of these genes or by regulating factors that modulate expression of these genes is under investigation. However, comparisons of the promoter regions of *relP*, *comX*, and *comYA* did not reveal any highly conserved regions that could serve as potential RcrR binding sites. In addition, on the basis of the behavior of particular mutants, we tend to favor the idea that the effects on *relP* and *com* gene expression are indirect.

The induction of competence is a specifically timed event linked to the accumulation of peptide signals and environmental stresses. Studies done with *S. mutans* have shown that acid tolerance, a key virulence factor of *S. mutans*, and competence are intimately linked. In particular, mutants lacking components of the competence signaling pathway had a diminished ability to grow at low pH (23). The simplest description of the model for development of competence in some streptococci involves quorum sensing of a secreted peptide by a two-component system, which triggers a cascade of expression of early and late competence genes involved in DNA uptake and recombination. However, recent studies reveal that the mechanisms underlying the regulation of the competence pathway in *S. mutans* are more complex and that much remains to be understood about the signals, signal transduction systems, and additional regulatory pathways affecting competence (5). For example, previous work done in our lab revealed that multiple signals are required for efficient activation of competence genes through the CiaRH and ComED two-component systems. ComED is the major TCS that regulates competence by sensing CSP levels, whereas CiaRH appears to be involved in sensing as-yet-undefined signals in a CSP-independent manner. It has been shown that *comYA* and *comX* are highly induced only when both CSP and horse serum (HS) are present, when levels of induction are compared to those achieved by the addition of CSP or HS alone, indicating that signals present in HS, possibly additional peptides, regulate competence genes in conjunction with CSP (5). In work done by another group, it has been shown that there are additional pathways that are independent of *comED* that are affecting transcription of *comX* and transformation (36, 37), suggesting that multiple parallel systems regulate the competence network in *S. mutans*.

The work presented here adds a novel dimension to the control of genetic competence in *S. mutans*, and likely in other naturally competent streptococci, by demonstrating that the RcrRPQ system plays a dominant role in modulating *com* gene expression and transformation in *S. mutans.* Notably, even after the addition of HS and CSP, no transformants could be obtained with the Δ*835np* mutant strain. The transformation deficiency of the Δ*835np* strain was associated with decreased expression of *comYA* and aberrant regulation of *comX* (Fig. 6). The addition of *SMu0835* (*rcrR*) back into the Δ*835np* and Δ*835p* strains restored wild-type levels of *comX* and *comYA* expression and wild-type transformation efficiency, whereas complementation of the Δ*835-837np* strain did not restore transformability. One explanation for the lack of transformation in the *835*$^{+}$/Δ*835-837np* strain could be due to the expression levels of *rcrR*. In the *835*$^{+}$/Δ*835-837np* stain, *rcrR* was not expressed at wild-type levels but was actually overexpressed when it was present on plasmid pDL278 (see Fig. S1A in the supplemental material). Interestingly, we also constructed a strain in which we overexpressed *rcrR* in a wild-type genetic background (Table 1; see Fig. S1A in the supplemental material). In this case, reductions in transformation efficiency (data not shown) and *comYA* expression (see Fig. S3 in the supplemental material) were noted. Therefore, the complete lack of transformability in the *835*$^{+}$/Δ*835-837np* complemented strain versus the behavior of the strain lacking only the ABC porter genes (Δ*835-837np*) may be related to overexpression of *rcrR*. Collectively, then, the data support the idea that RcrR has the ability to influence the expression of the *com* genes and transformation independently of the ABC transporters.

We propose that the way in which RcrR impacts competence is by negatively regulating a factor that interferes with ComX activation of *comY*. Specifically, it is established that ComX is required for activation of *comYA* expression in response to CSP. However, particular mutants lacking RcrR (Fig. 6) display upregulation of *comX* with concurrent downregulation of *comY*. The simplest explanation for these findings is that RcrR represses the expression of an anti-sigma factor, similar to the model in *Streptococcus pneumoniae* in which ComX activity is negatively controlled by the ComW anti-sigma factor, blocking activation of late *com* gene expression (28, 45). Interestingly, in *Streptococcus salivarius*, a cytoplasmic Rgg-like regulator, ComR, and a small hydrophobic peptide, ComS, were found to be necessary for the induction of *comX* and transformation, and ComS internalization was dependent on an oligopeptide transport system (15). Recently, an analogous ComRS systems was described in *S. mutans* (30). Our preliminary analysis confirmed that ComRS is required for competence development in *S. mutans* and that a *comRS* mutant remained nontransformable in the *rcrR* polar mutant. Whether RcrRPQ modulate competence development by affecting the transcription or activities of ComRS remains to be determined.

The effect of the *rcr* system on *comY* and competence appears not only to be affected by RcrR but also to depend

strongly on the level of expression of the *rcrPQ* genes. In fact, one of the more intriguing aspects of this study is that defects in transformation are evident with both the loss of and the overproduction of the ABC porters. In particular, the Δ*835polar* mutant, which has no RcrR but which expresses levels of *rcrPQ* that are similar to those for the wild-type strain, for reasons explained in Results, has a phenotype opposite that of the Δ*835np* strain, which overexpresses the *rcrPQ* genes by >100-fold. In this case, the Δ*835p* polar mutant was hypertransformable and displayed upregulation of *comY*, whereas the Δ*835np* strain could not be transformed, even when exogenous CSP was provided, and had low levels of *comY* expression. Furthermore, the Δ*836p* polar mutant, which had lost both porters, had reduced transformation efficiency compared to that of the wild-type strain, reinforcing the suggestion that the appropriate expression of the exporters is necessary for efficient transformation. The complementation data with the *rcrR* gene provide further evidence that appropriate levels of the RcrPQ porters and proper regulation of the *rcrRPQ* operon are necessary for efficient transformation. Consequently, whatever the RcrPQ exporters are externalizing is necessary for proper regulation and development of competence. Of note, we tested the transformation efficiency of a Δ*comC* Δ*835p* double-knockout strain and observed a hypertransformable phenotype, as was seen in the strain carrying only the Δ*835p* mutation, so the RcrPQ porters are probably not affecting the production or secretion of ComC. Also, the signal(s) being exported by these transporters must have the potential to act from inside and/or outside the cell, since *rcrPQ* expression can be neither too high nor too low if efficient transformation of cells is to occur.

In summary, *S. mutans* has multiple pathways to cope with environmental stresses. An intimate linkage has already been established between stress tolerance and (p)ppGpp metabolism (20, 35) and stress and the induction of competence (23). In this study we show that the previously uncharacterized *rcrRPQ* operon provides a crucial yet complex linkage between oxidative and acid stresses, genetic competence, and (p)ppGpp metabolism. Operons that are similar to *rcrRPQ* are distributed in a variety of related bacteria (see Table S2 in the supplemental material), but their role in stress tolerance, (p)ppGpp metabolism, and competence has not yet been explored. The results outlined here indicate that RcrRPQ proteins have the potential to serve as master regulators of growth and stress tolerance in many bacteria. Disclosing the substrates for the porters and the signal(s) detected by the RelRS-like TCS may offer new opportunities to control biofilm growth and to modulate the virulence of a variety of important human pathogens.

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